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SAFETY TESTING OF DENGUE-1 AND DENGUE-3 SEEDS FOR HUMAN CHALLENGES, UNATTENUATED

PHASE REPORT

LOUIS POTASH

March 16, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Dengue Virus Type 3 (non-attenuated) Strain: CH-53489, LOT No. 1 of April 1984 was satisfactorily safety-tested in accordance with the guidelines established by the FDA for live and inactivated vaccines, as stipulated in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10 - 630.18, etc. All testing procedures were carried out following Good Laboratory Practices regulations (21 CFR, Part 58).

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publications No. (NIH) 78-23, Revised 1978).

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I. INTRODUCTION

The accompanying protocol is a description of the safety testing of a lot of dengue virus type 3 designated as:

Dengue Virus Type 3 (Non-Attenuated)
Strain CH-53489

Utilizing the testing procedures herein described, this fluid is considered to have passed satisfactorily all tests for safety including purity. The detailed records with respect to passage history, pool production, final product, virus characterization and subsequent safety testing may be found in the laboratory notebooks located at:

The Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100 - (Dr. Ken Eckels)

The Experimental Virus Vaccine Production Laboratory - Suite #500 - Flow Laboratories, Inc., McLean, VA - (Dr. Louis Potash)

All procedures performed at Flow Laboratories followed Good Laboratory Practices regulations (21 CFR, Part 58) and were carried out in accordance with the guidelines established by the FDA for live and inactivated vaccines as found in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10 - 630.18, etc. These procedures are detailed in the following SOPs and recorded on the indicated VVPL Forms:

SOP No.:	400.002 -	Issued	25 Feb 1980,	Revised	18 Feb	1986
	400.004 -	"	25 Feb 1980,	"	18 Feb	1986
	400.005 -	"	25 Feb 1980,	"	18 Feb	1986
	400.006 -	"	25 Feb 1980,	"	18 Feb	1986
	400.007 -	"	25 Feb 1980,	"	18 Feb	1986
	400.008 -	"	12 Apr 1984,	"	18 Feb	1986
	400.009 -	"	3 May 1984,	"	18 Feb	1986
	500.001 -	"	29 Oct 1980,	"	18 Feb	1986
	500.002 -	"	29 Oct 1980,	"	18 Feb	1986
	500.008 -	"	13 Jan 1981,	"	3 Mar	1986
	500.009 -	"	23 Feb 1981,	"	3 Mar	1986
VVPL FORM	#001 -	Issued	25 Feb 1981,	Revised	2 Mar	1984
	003 -	"	3 Apr 1984			
	004 -	"	16 Jan 1981,	"	21 Mar	1984
	008 -	"	29 Oct 1980,	"	3 May	1984
	015 -	"	15 Jan 1981,	"	13 July	1984
	016 -	"	15 Jan 1981,	"	13 July	1984
	017 -	"	16 Jan 1981,	"	13 Jan	1986

II. SYNOPSIS

A. Virus Strain: Dengue Virus Type 3 (Non-Attenuated) Strain: CH-53489

B. Live Virus Vaccine Pool Designation: MFG Date: April 1984, LOT No. 1

C. Treatment/Handling: Freeze-Dried: Rehydrate to 3 ml with Sterile Distilled Water

D. Safety Tests on Crude Harvest Fluids:

1. Sterility: Fluid Thioglycollate (FTM), Trytone Soya Broth (TSB), Lowenstein-Jensen Egg Medium, Mycoplasma

a.	Virus Infected Fluid	(52 ml)	No Growth
b.	Control Fluid (TCF)	(52 ml)	No Growth

2. Tissue Culture Identity and Purity (Safety): AGMK, PHA, FRhL-2, PRK, and Flow 5000.

a.	Virus Infected Fluid	(92 ml)	Satisfactory*
b.	Control Fluid (TCF)	(92 ml)	Satisfactory

3. Animal Safety:

a. Adult Mice: Intracerebral and I.P.

(1)	Virus Infected Fluid	(11 ml)	Satisfactory
(2)	Control Fluid (TCF)	(11 ml)	Satisfactory

b. Suckling Mice: Intracerebral and I.P.

(1)	Virus Infected Fluid	(2.5 ml)	Satisfactory
(2)	Virus Fluid Neutralized	(2.5 ml)	Satisfactory
(3)	Control Fluid (TCF)	(2.5 ml)	Satisfactory

c. Guinea Pigs: Intracerebral and I.P.

(1)	Virus Infected Fluid	(15.5 ml)	Satisfactory
(2)	Control Fluid (TCF)	(15.5 ml)	Satisfactory

d. Rabbits: Intradermal and Subcutaneous

(1)	Virus Infected Fluid	(20 ml)	Satisfactory**
(2)	Control Fluid (TCF)	(20 ml)	Satisfactory**

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* Complete inhibition of the Coxsackie A-9 challenge virus was observed in repeated tests in AGMK in those tubes inoculated with 14-day harvest fluids derived from cultures initially inoculated with serum/virus mixtures.

** One rabbit from each group died after initial 21 days observation with deaths attributed to intestinal blockages.

E. Final Product Testing:

1.	Microbial Sterility: Fluid Thioglycollate & Soybean-Casein Digest Media	(20 x 3 ml vials)	No Growth
2.	Reverse Transcriptase:	(1 ml)	No RT Enzyme
3.	General Safety:		
	a. Mice: I.P.	(2 x 0.5 ml)	Satisfactory
	b. Guinea Pigs: I.P.	(2 x 5.0 ml)	Satisfactory

III. DETAILED SUMMARY RELATING TO THE SAFETY TESTING OF A LOT OF DENGUE VIRUS TYPE 3 (NON-ATTENUATED) STRAIN: CH-53489, PROPAGATED IN DBS-FRhL-2 CELL CULTURES

A. Inocula

On June 6, 1986, the following frozen materials were obtained for testing from Dr. K. Eckels, Contracting Officer's Representative, at the Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100.

1. Dengue-3 challenge seed, d9 harvest, unclarified of 25 Apr 1984: 10 x 20 ml vials

2. WR-FRhL-1, control fluid for above, d9 harvest, unclarified of 25 Apr 1984: 10 x 20 ml vials

3. Dengue-3 Strain CH-53489, non-attenuated, final product of April 1984, LOT No.1: 24 x 3ml freeze-dried.

4. Antiserum: Dengue-3, M-HAF, H-37, 6-17: 2 x 5 ml vials.

On arrival in this laboratory, the materials were stored as follows: Items #1 and #2 at -70°C, or below; Items #3 and #4 at -20°C, or below.

B. Safety Testing Procedures and Results on the Crude, Unclarified Harvest Fluids (SOP No.: 500.008)

1. Microbial Sterility - (VVPL FORM #011)

Aliquots of the bulk frozen fluids were thawed and tested for microbial sterility as follows:

a. Fluid Thioglycollate Medium - FTM - (LOT #35045204): Each of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluid and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 31°C (± 1°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 30 culture tubes.

b. Trytone Soya Broth - TSB - (LOT #35060207): Each of 10 culture culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluid and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 22°C (± 2°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 30 culture tubes.

c. Lowenstein-Jensen Egg Medium (DIFCO - Lot #741692): Each of 10 culture tubes was inoculated with 0.5 ml of the crude virus fluid and each of 10 culture tubes was inoculated with 0.5 ml of the crude control fluid. Ten additional culture tubes were included as uninoculated controls. All cultures were incubated at 37°C -- horizontally for the first 24 hours and then vertically for the remainder of the 8-week observation period. Cultures were examined periodically for growth over this 8-week period. No growth was observed in any of the cultures.

The results of the above described microbial sterility assays are summarized in Table I.

d. Mycoplasma Sterility: These assays were performed by the Mycoplasma Testing Section of the Flow Laboratories' Quality Control Laboratory and included both the routine PPLO agar and broth assays and the specific test for the detection of M. hyorhinis. Samples (1 x 25 ml and 1 x 2 ml) of both the crude virus and control fluids were submitted for testing. The samples were reported to be negative for mycoplasmas. A copy of their report is appended to this Protocol. (Appendices - 1 and 2).

2. Identity in Tissue Culture (Serum-Neutralization) - (VVPL FORM #015)

An attempt to identity the crude virus pool was carried out using AGMK tube cultures. Equal volumes of the crude virus pool and a 1:50 dilution of the immune serum (Den-3, M-HAF, H-17, 6-17) were mixed and incubated at room temperature for 60 minutes. To each of 4 tissue culture tubes was added 0.4 ml of the serum-virus mixture. In addition, to each of 4 tubes was added 0.2 ml of either the undiluted crude virus fluid or the undiluted immune serum. Four culture tubes were included as uninoculated cell lot controls. Prior to inoculation all AGMK tube cultures were refed with 2 ml of Medium MEM containing 5% fetal bovine serum (heat inactivated) plus antibiotics - (VVPL-BM-833-1-3). Cultures were incubated at 35°C for 7 days at which time no CPE was detected in any of the cultures. Two cultures from each set were tested for hemadsorption - medium was decanted and films were overlayed with 1 ml of 0.1% guinea pig RBC (in PBS) with incubation at 4°C for a minimum of 30 min. Films were examined microscopically for hemadsorption - all were negative. Two cultures from each set were fixed and stained with a solution containing 5% glutaraldehyde + 0.025% crystal violet. Microscopic examinaton of the films confirmed the absence of any discernible CPE.

Identity Test Results:

Inoculum	AGMK Cell Lot #65284 CPE	Hads	Stain
Serum-virus mixture	0/4	0/2	0/2
Virus alone - control	0/4	0/2	0/2
Serum alone - control	0/4	0/2	0/2
Cell lot control	0/4	0/2	0/2

3. Purity (Safety) in Tissue Cultures - (VVPL FORM #016)

a. Tissue Cultures: Cell cultures were obtained as fully "sheeted" flask or roller tube cultures from Flow Laboratories' Tissue Culture Department. Cultures were maintained on Medium MEM containing 2 to 10% fetal bovine serum (heat-inactivated) plus antibiotics: gentamicin, 100 mcg/ml; neomycin, 50 mcg/ml; and amphotericin B (I.V.), 2.5 mcg per ml. Cultures were inoculated, refed and subpassaged as indicated below. The following tissue culture systems were utilized:

(1)	Tertiary African Green Monkey Kidney (AGMK)	MEM + 5% serum
(2)	Primary Human Amnion (PHA)	MEM + 10% serum
(3)	Fetal Rhesus Lung (FRhL-2)	MEM + 5% serum
(4)	Primary Rabbit Kidney (PRK)	MEM + 5% serum
(5)	Whole Human Embryo Fibroblast (Flow 5000)	MEM + 5% serum

b. General Testing Procedures

(1) Crude Virus Fluid

(a) Primary Flask Cultures: Equal volumes of the bulk crude virus fluid and a 1:50 dilution of the immune serum (Den-3, M-HAF, H-87, 6-17) were well mixed and incubated at room temperature for 60 minutes. A total of 15 ml of the virus fluid was tested per tissue culture system wherein each of 2 - 75 cm^2 flasks per tissue culture system was inoculated with 15 ml of this serum-virus mixture. Flasks contained approximately 25 ml of maintenance medium at the time of inoculation. Cultures were incubated at 35°C (37°C for PHA) for 14 days with periodic microscopic examination for any signs of CPE and/or cellular degradation. When necessary to maintain the integrity of the cell films, cultures were refed with 35 ml of fresh medium.

(b) Secondary Tube Subcultures: On the 14th day of incubation, the primary cultures were re-examined microscopically and the fluids harvested individually and treated with the specific immune serum - 0.1 ml per harvest. In addition, to each individual harvest was added: 0.1 ml gentamicin (50 mg/ml); 1 ml penicillin- streptomycin solution (5000 units/ml and 5000 mcg/ml, respectively); and 10% of 10X SPG* (v/v). Following mixing, the fluids were incubated at room temperature for 60 minutes and then subpassed into homologous roller tube cultures - 0.5 ml of each harvest into each of 20 tubes. The remainder of the harvest fluids was saved and stored at -75°C, or below. All primary cultures were tested for hemadsorption by the addition of 0.1% guinea pig RBC (in PBS) and incubation at 4°C for a minimum of 30 minutes. All cultures were negative for hemadsorption.

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* 10X SPG: sucrose, 2.18 M; KH_2PO_4, 0.038 M; K_2HPO_4, 0.072 M; potassium glutamate, 0.049 M.

Tube cultures (refed with 2 ml of maintenance medium prior to inoculation) were incubated at 35°C (37°C for PHA) for 14 additional days. When necessary to maintain the integrity of the cell films, cultures were refed with 2 ml of fresh medium. Cultures were examined microscopically at periodic intervals and at the end of the incubation period for any signs of CPE. After final examination, tubes were divided - depending on the specific cell system - for additional testing:

AGMK, PHA, FRhL-2 and Flow 5000 Tube Cultures: These were divided into 3 groups as follows:

1/4th tested for the presence of hemadsorbing agents,
1/4th fixed and stained with a solution of 5% glutaraldehyde + 1:10 giemsa stain and examined microscopically for any CPE,
1/2 Challenged with Coxsackie A-9 virus (0.2 ml per tube at the dilutions noted in the tables) for the detection of non-CPE producing agents and/or latent agents via the interference phenomenon.

PRK Tube Cultures: These were equally divided into 2 groups:

1/2 tested for the presence of hemadsorbing agents,
1/2 fixed and stained with the glutaraldehyde-giemsa stain solution and examined microscopically for any CPE.

No challenge studies were carried out with the Coxsackie A-9 virus since this virus does not produce any discernible CPE in this tissue culture system.

(2) Crude Control Fluid

Equal volumes of the crude control fluid and the indicated maintenance medium were well mixed and incubated at room temperature for 60 minutes. A total of 15 ml of the control fluid was tested per tissue culture system wherein each of 2 - 75 cm^2 flasks per tissue culture system was inoculated with 15 ml of the above mixture. Cultures were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included.

(3) Uninoculated Cell Lot Controls

Two 75-cm^2 flasks or bottles per tissue culture system were included as uninoculated cell lot controls and were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included. In addition, an appropriate number of uninoculated roller tube cultures were included as cell lot controls for the secondary tube subcultures.

In all challenge studies, 1 to 4 culture tubes per set were left unchallenged to serve as controls to the challenge virus.

The results of these in vitro Tissue Culture Purity (Safety) tests are summarized in Tables II-A through -E.

4. Animal Safety Tests - (VVPL FORM #004)

a. Adult Mice - Test for adventitious agents - (SOP No. 400.005)

Each of 20 adult CD-1 mice (15-20 grams each) was inoculated intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml of the un-neutralized crude virus fluid and each of 20 adult CD-1 mice was similarly inoculated with the crude control fluid. An additional 10 mice were included as uninoculated controls. The mice were observed daily for deaths and/or signs of illness or distress over a 4 week period. All mice (inoculated as well as controls) remained healthy and survived the entire 28-day observation period with no evidence of lymphocytic choriomeningitis virus infection or of any other virus infection. This test in adult mice was considered satisfactory.

b. Suckling Mice - Test for adventitious agents - (SOP No.: 400.005)

Three groups of 20 newborn CD-1 mice from mixed litters (10 per mother - less than 24 hours old) were inoculated intracerebrally with 0.01 ml and intraperitoneally with 0.1 ml as follows: one group with un-neutralized crude virus fluid; one group with neutralized virus fluid (0.1 ml undiluted antiserum + 2.5 ml crude virus); and one group with the crude control fluid. An additional litter of 10 sucklings was included as uninoculated controls. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. One suckling inoculated with the control fluid was found cannibalized within the first 24 hours. There were no other deaths and none of the sucklings exhibited any signs of illness or distress over the initial 14-day observation period.

On the 14th day, single pools were prepared of the emulsified tissue (minus skin and viscera) of the following groups: a) un-neutralized virus inoculated sucklings (20); b) neutralized virus inoculated sucklings (20); c) control fluid inoculated sucklings (19); and d) uninoculated controls (10). A blind passage into newborn CD-1 mice was made of each of the 4 pools via the intracerebral and intraperitoneal routes: the individual pools from the inoculated sucklings (a, b and c) into each of 20 newborns and the pool from the uninoculated control sucklings (d) into 10 newborns. An additional litter of 10 sucklings was included as uninoculated controls (e) for this blind passage. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. Of the sucklings inoculated with pool 'b' (derived from the neutralized virus inoculated group), one (1) was found cannibalized within the first 24 hours. There were no other deaths and none of the sucklings exhibited any signs of illness or distress over this final 14-day observation period.

Since none of the inoculated sucklings (neutralized virus, un-neutralized virus or control fluid) exhibited any evidence of a transmissible agent or of Coxsackie virus infection or of any viral infection, and since 100% of the these inoculated sucklings remained healthy and survived the entire observation period, this test in suckling mice was considered satisfactory.

c. Adult Guinea Pigs - (SOP No.: 400.006)

Test for M. tuberculosis: Each of 3 adult guinea pigs (Hartley Strain, virus free, 350-450 grams each) was inoculated intracerebrally with 0.1 ml and intraperitoneally with 5 ml of the un-neutralized crude virus fluid, and each of 3 guinea pigs was similarly inoculated with the crude control fluid. An additional 3 guinea pigs were included as uninoculated controls. All pigs were observed daily for a period of 6 weeks for deaths and/or any signs of illness or distress. One guinea pig inoculated with the control fluid was found dead within the first 24 hours; however, there were no other deaths nor signs of illness or distress. Commencing on day 21, daily rectal temperatures (LED digital thermistor thermometer) were taken and recorded (± 1300 hrs) for all guinea pigs until time of sacrifice. The average temperatures ($^{\circ}$C) for the 3 groups of guinea pigs were: for the virus fluid inoculated - 38.60, 38.65, and 38.65; for the control fluid inoculated - 38.61 and 38.71; and for the uninoculated controls - 38.57, 38.64 and 38.65. There were no significant rises indicative of either bacterial or viral infection. All guinea pigs appeared healthy and survived the entire 42-day observation period at which time they were necropsied following euthanasia with Halathane. Inspection of the abdominal and thoracic cavities indicated no gross pathological changes. This test in guinea pigs was considered satisfactory.

d. Adult Rabbits - Test for B-virus and other adventitious agents (SOP No.: 400.004)

Each of two New Zealand white rabbits (1500-2500 grams each) was inoculated intradermally in multiple sites with a total of 1.0 ml and subcutaneously with 9.0 ml with the un-neutralized crude virus fluid. In addition, the left cornea was scratched and 0.03 ml of the virus fluid was applied. Two rabbits were similarly inoculated with the crude control fluid but with the right cornea scratched. One additional rabbit was included as an uninoculated control. All rabbits were observed daily for a total of 28 days for deaths and/or signs of lesions at sites of inoculation and for any signs of illness or distress. All rabbits remained healthy and none exhibited any signs of illness or distress or lesions at the sites of inoculation for at least 21 days. On day 21, one of the rabbits inoculated with the control fluid not only stopped eating and drinking but stopped defecating and urinating at the same time. In spite of undergoing recommended treatment (administration of ampicillin and laxotone), this animal was found dead on day 23. Necropsy of this rabbit suggested that intestinal blockage was the most probable cause of death as there were no other gross pathological indications. On day 25, one of the rabbits inoculated with the virus fluid exhibited the same symptoms and, in spite of treatment, was found dead on day 27. Necropsy of this animal also suggested that intestinal blockage was the most probable cause of death as there were no other gross pathological indications. As all rabbits did survive and remain healthy for 21 days (the normal observation period for rabbits as indicated in CFR 21, Part 630.16), this test was considered satisfactory.

The results of these in vivo Animal Safety Tests are summarized in Table III - A and - B.

C. Final Product Testing and Results - (SOP No.: 500.009)

1. Microbial Sterility

A total of 20 x 3 ml vials of the freeze-dried final virus product was submitted to Ben-Venue Laboratories, Inc., for microbial sterility testing via the Membrane Filtration Method in Fluid Thioglycollate and Fluid Soybean-Casein Digest Media. No growth was reported and a copy of their report is appended to this Protocol - (Appendixes - 3 and 4).

2. Reverse Transcriptase - Assay for the detection of RNA-dependent DNA-polymerase activity

The assay for Reverse Transcriptase was performed by Dr. Allan Tereba at the St. Jude Children's Research Hospital, Memphis, TN. A 1.0 ml sample of the reconstituted freeze-dried virus fluid and a 2 ml sample of the clarifed (centrifuged) control fluid were submitted for assay. Both samples were reported to be negative for the RT Enzyme and a copy of this report is appended to this Protocol - (Appendixes - 5 and 6).

3. General Safety Test - (SOP No.: 400.002 - VVPL FORM #001)

Each of 2 overtly healthy CD-1 mice (less than 22 grams each) and each of 2 overtly healthy guinea pigs (Hartley Strain, virus free - less than 400 grams each) were inoculated intraperitoneally with 0.5 ml and 5 ml, respectively, of the reconstituted freeze-dried final virus product. Two additional animals of each species were included as uninoculated controls. All animals were weighed prior to inoculation and on day 7 post inoculation. All animals were observed daily over this 7-day period for deaths and/or signs of illness or distress - none were noted. All animals remained healthy and all exhibited weight gains. This test was considered satisfactory. The results of these General Safety Tests are summarized in Table IV.

Table I. Microbial Sterility Test Results on the Crude Dengue-3 Virus Seed for Human Challenge, Unattenuated.

Culture Medium	No.	Vol. per culture (ml)	Temperature	Date On Test	Date Off Test	Results
Fluid Thioglycollate						
(FTM) LOT #35045204	10	———	30-32°C	7/21/86	8/11/86	No Growth
Virus Infected Fluid	10	1.0		7/21/86	8/11/86	No Growth
Control Fluid	10	1.0		7/21/86	8/11/86	No Growth
Tryptone Soya Broth						
(TSB) LOT #35060207	10	———	22°C	7/21/86	8/11/86	No Growth
Virus Infected Fluid	10	1.0		7/21/86	8/11/86	No Growth
Control Fluid	10	1.0		7/21/86	8/11/86	No Growth
Lowenstein-Jensen Egg						
Medium - LOT #741692	10	———	37°C	7/21/86	9/15/86	No Growth
Virus Infected Fluid	10	0.5		7/21/86	9/15/86	No Growth
Control Fluid	10	0.5		7/21/86	9/15/86	No Growth

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue - 3 Seed Virus for Human Challenge, Unattenuated

A. Tertiary African Green Monkey Kidney (AGMK) - Initial Assay

	Initial Flasks Lot # 65303 Day 14			0.5 ml per tube Passage #1 Lot # 65358 Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE **	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	***		***	#		#				
Virus/Serum Mixture	2/2	0/2	2/2	40/40	0/10	10/10	0/4	0/4	0/4	0/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	3/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	2/4
Control - (2)				0/52	0/12	0/12	6/6	6/6	6/6	6/6

* Coxsackie A-9 Challenge Results based on a 5-day incubation at 35°C.

** On day 7, all flasks refed with 35 ml of fresh medium. The flasks inoculated with the virus/serum mixture exhibited morphological changes possibly related to virus breakthrough and were, therefore, treated with 0.? ml of undiluted immune serum.

*** By day 14, films exhibited a non-descript CPE confirmed on staining - attributed to Dengue-3 virus breakthrough.

By day 21 (day 14 + 7), films again exhibited the same non-descript CPE which by day 28 (day 14 + 14) was more manifested. These films interfered with the Coxsackie A-9 challenge thereby necessitating a repeat study - the 14-day subpass.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-3 Seed Virus for Human Challenge, Unattenuated

A. Tertiary African Green Monkey Kidney (AGMK) - First Repeat 14-day Subpass Assay

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 65303			Freeze #2117 p3						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE**	Hads	Stain	CPE#	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	***		***	##		##				
Virus/Serum Mixture	2/2	0/2	2/2	40/40	0/10	0/10	0/4	0/4	0/4	0/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	2/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	1/4
Control - (2)				0/47	0/12	0/12	5/5	5/5	5/5	5/5

* Coxsackie A-9 Challenge Results based on a 5-day incubation at 35°C.

** On day 7, all flasks refed with 35 ml of fresh medium. The flasks inoculated with the virus/serum mixture exhibited morphological changes possibly related to virus breakthrough and were, therefore, treated with 0.2 ml of undiluted immune serum.

*** By day 14, films exhibited a non-descript CPE confirmed on staining - attributed to Dengue-3 virus breakthrough.

Forty-eight hours prior to the repeat day 14 subpassage, those 40 tubes to be inoculated with the virus/serum flask harvests were pre-treated with 0.2 ml of a 1:50 dilution of the immune serum. On day of subpassage, 0.1 ml of the immune serum was added to the 14-day harvests (35 day storage at -70°C) and allowed to incubate at room temperature for 1 hour prior to subpassage.

By day 23 (day 14 + 9), films again exhibited the same non-descript CPE which did not intensify by day 28 (day 14 + 14). These films again completely inhibited the Coxsackie A-9 challenge leading to a second a repeat study with the same 14-day harvest fluids.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-3 Seed Virus for Human Challenge, Unattenuated

A. Tertiary African Green Monkey Kidney (AGMK) - Second Repeat 14-day Subpass Assay

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 65303			LOT #65545						
	Day 14			Day 14 + 13 = 27						
							Coxsackie A-9 Challenge*			
Material Tested	** CPE	Hads	Stain	# CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	2/2 ***	0/2	2/2 ***	0/40	0/10	0/10	0/4	0/4	0/4	0/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control - (2)				0/50	0/12	0/12	6/6	6/6	6/6	6/6

* Coxsackie A-9 Challenge Results based on a 6-day incubation at 35°C.

** On day 7, all flasks refed with 35 ml of fresh medium. The flasks inoculated with the virus/serum mixture exhibited morphological changes possibly related to virus breakthrough and were, therefore, treated with 0.2 ml of undiluted immune serum.

*** By day 14, films exhibited a non-descript CPE confirmed on staining - attributed to Dengue-3 virus breakthrough.

Twenty-four hours prior to the repeat day 14 subpassage, those 40 tubes to be inoculated with the virus/serum flask harvests were pre-treated with 0.2 ml of a 1:10 dilution of the immune serum. On the day of subpassage, 0.5 ml of the immune serum was added to the 14-day harvests (111 day storage at -70°C) and allowed to incubate at 37°C for 2 hours prior to subpassage.

Although no morphological changes were observed in the virus/serum inoculated tubes, these tubes again completely inhibited the Coxsackie A-9 challenge virus.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-3 Seed Virus for Human Challenge, Unattenuated

A. Tertiary African Green Monkey Kidney (AGMK) - Repeat Initial Assay

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 833-007			LOT # 833-019						
	Day 15			Day 15 + 13 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	2/2**	0/2	2/2	0/40	0/10	0/10	0/4	0/4	0/4	0/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control - (1)	0/2	0/2	0/2	0/39	0/10	0/10	4/4	4/4	4/4	4/4
Control - (2)				0/52	0/12	0/12	6/6	6/6	6/6	5/6

* Coxsackie A-9 Challenge Results based on a 4-day incubation at 35°C. Prior to challenge tubes refed with 2 ml of fresh medium.

** Prior to inoculation, virus + serum (1:16 dilution) incubated at 37°C for 2 hours. By day 9, these flasks inoculated with the virus/serum mixture exhibited morphological changes which by day 15 were quite extensive - attributed to virus breakthrough. Day 15 harvests were treated with 0.3 ml of undiluted immune serum with incubation at 37°C for 2 hours prior to subpassage.

Although no morphological changes were observed in the virus/serum inoculated tubes, these tubes again completely inhibited the Coxsackie A-9 challenge virus.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue - 3 Seed Virus for Human Challenge, Unattenuated

B. Primary Human Amnion (PHA)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 65341			Lot # 65467						
	Day: 14			Day: 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE **	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	3/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	1/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control - (2)				0/59	0/12	0/12	8/8	8/8	8/8	7/8

* Coxsackie A-9 Challenge Results based on a 7-day incubation at 37°C. All challenged tubes refed with 2 ml of fresh medium prior to challenge.

** On day 25 (14 + 11), all tubes were refed with 2 ml of fresh medium.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue - 3 Seed Virus for Human Challenge, Unattenuated

C. Fetal Rhesus Lung (FRhL-2)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 65284 p21			Lot # 65396 p25						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	** CPE	Hads	Stain	*** CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	0/2	0/2	ND	0/40	0/10	0/10	4/4	4/4	3/4	1/4
Control Fluid (TCF)	0/2	0/2	ND	0/40	0/10	0/10	4/4	4/4	4/4	1/4
Control - (1)	0/2	0/2	ND	0/39	0/10	0/10	4/4	4/4	4/4	2/4
Control - (2)				0/50	0/12	0/12	6/6	6/6	6/6	0/6

* Coxsackie A-9 Challenge Results based on a 4-day incubation at 35°C. All challenged tubes refed with 2 ml of fresh medium prior to challenge.

** On day 9, all films exhibited early signs of cellular degeneration and flasks were refed with 35 ml of fresh medium. In addition, to the flasks originally inoculated with the virus/serum mixture was added 0.2 ml of undiluted immune serum. By day 14, all films exhibited similar degrees of cellular degeneration. Films were not fixed and stained.

*** On day 23 (day 14 + 9), all tubes were refed with 2 ml of fresh medium. No cellular degeneration was detected in the tube cultures.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-3 Virus Seed for Human Challenge, Unattenuated

D. Primary Rabbit Kidney (PRK)

	Initial Flasks			0.5 ml per tube Passage #1		
	Lot # 65267			Lot # 65347		
	Day: 14			Day: 14 + 14 = 28		
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain
Virus/Serum Mixture	0/2	0/2	0/2	0/40	0/20	0/20
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/20	0/20
Control - (1)	0/2	0/2	0/2	0/40	0/20	0/20
Control - (2)				0/24	0/12	0/12

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue - 3 Seed Virus for Human Challenge, Unattenuated

E. Whole Human Embryo Fibroblasts (Flow 5000)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 65280 p19			Lot # 65354 p17						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	0/2	0/2	0/2	0/39	0/10	0/10	4/4	4/4	4/4	0/4
Control Fluid (TCF)	0/2	0/2	0/2	0/39	0/10	0/10	4/4	4/4	4/4	1/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	3/4	1/4
Control - (2)				0/52	0/12	0/12	6/6	6/6	6/6	4/6

* Coxsackie A-9 Challenge Results based on a 7-day incubation at 35°C.

Table III - A. Animal Safety Tests Results on the Crude Dengue-3 Virus Seed for Human Challenge, Unattenuated

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Mice (15-20 grams)	Virus Pool Un-neutralized	0.03 0.50	I. Cer. I.P.	20	No deaths nor signs of illness or distress recorded.	Test Satisfactory
	Control Fluid (TCF)	0.03 0.50	I. Cer. I.P.	20		
	None	—	—	10		
Suckling Mice (< 24 hours)	Virus Pool Neutralized	0.01 0.10	I.Cer. I.P.	20	Control Fluid (TCF): one suckling found cannibalized within first 24 hours.	Test Satisfactory
	Virus Pool Un-neutralized	0.01 0.10	I.Cer. I.P.	20		
	Control Fluid (TCF)	0.01 0.10	I.Cer. I.P.	20	No other deaths nor signs of illness or distress over this initial 14-day period.	
	None	—	—	10		
	D14 Blind Passage (VP-N)	0.01 0.10	I. Cer. I.P.	20	VP-N Group: one suckling found cannibalized within first 24 hours.	100% survival of inoculated sucklings. No evidence of a transmissible agent or of any viral infection.
	D14 Blind Passage (VP-Un)	0.01 0.10	I. Cer. I.P.	20		
	D14 Blind Passage (TCF)	0.01 0.10	I.Cer. I.P.	20	No other deaths nor signs of illness or distress over this final 14-day period.	
	D14 Blind Passage (None)	0.01 0.10	I.Cer. I.P.	10		
	D14 - None	—	—	10		

Table III - B. Animal Safety Tests Results on the Crude Dengue-3 Virus Seed for Human Challenge, Unattenuated

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Guinea Pigs (350-450 gms)	Virus Pool Un-neutralized	0.10 5.00	I. Cer. I.P.	3	One Control Fluid inoculated guinea pig found dead within first 24 hours. No other deaths nor signs of illness or disease. Daily rectal temperatures taken over last 3 weeks of observation were within normal ranges.	
	Control Fluid (TCF)	0.10 5.00	I.Cer. I.P.	3		
	None	—	—	3		

Code	GP#	Mean Temp. (°C)	Temp. Range (°C)
VP-1	423	38.65	38.3 - 39.1
VP-2	424	38.60	38.2 - 38.9
VP-3	425	38.65	38.2 - 38.9
TCF-1	421	38.71	38.3 - 38.9
TCF-2	422	38.61	38.3 - 39.0
C-1	418	38.64	38.1 - 38.9
C-2	419	38.57	38.1 - 38.8
C-3	426	38.65	38.3 - 39.1

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Rabbits (1500-2500 gms)	Virus Pool Un-neutralized	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. L.Cornea	2	One control inoculated rabbit found dead on day 23 and one virus inoculated rabbit found dead on day 27. Both rabbits had stopped eating and drinking as well defecating & urinating 2 days prior to death. Necropsy findings suggested that intestinal blockage was the most probable cause of death as there were no other gross pathological indications. There were no other signs of illness or distress and no lesions at sites of inoculation.	
	Control Fluid (TCF)	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. R. Cornea	2		
	None	—	—	1		

Table IV. General Safety Test Results on the Final Product of Dengue-3 Seed Virus for Human Challenge, Unattenuated

Animal Species	Inoculum	Vol. (ml)	Tag #	Weight in Grams Day 0	Weight in Grams Day 7	Weight Gain/ (Loss) in Grams
Mice	Dengue-3	0.5	263	21.2	27.6	6.4
			264	18.8	24.3	5.5
	None	---	265	21.0	25.8	4.8
			266	21.3	27.5	6.2
Guinea Pigs	Dengue-3	5.0	416	390.1	424.1	34.0
			417	366.4	402.8	36.4
	None	---	418	354.9	394.4	39.5
			419	382.1	397.6	15.5

7655 Old Springhouse Road
McLean, Virginia 22102. USA
(703) 893-5925

Flow Laboratories, Inc.
A Flow General Company

September 10, 1986

Dr. Louis Potash
Flow Labs., Inc.
McLean, VA

Charge #833/8340

Dear Dr. Potash:

Your four samples:

1. Dengue-1 virus seed
2. Dengue-1 control fluid
3. Dengue-3 virus seed
4. Dengue-3 control fluid

submitted for the presence of Mycoplasma hyorhinis using direct immunofluorescence staining and the DNA Hoechst stain and agar testing were found to be negative.

Sincerely,

Jim Quartey
Mycoplasma Lab

JQ:wsb

MYCOPLASMA TEST RECORD SHEET

Culture Medium	LOT #	No. ml Tested		Date		Results
		Aerobic	Anaerobic	On Test	Off Test	
Virus Fluid - LOT # DENGUE-3 - QC 535						
PPLO Agar	B0063119	.2	.2	8/11/86	8/26/86	NEGATIVE
PPLO Broth	3E0062164	.25	—			NEGATIVE
D 5 Subpass to Broth		.25	—	8/18/86	9/2/86	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		.25	.25	8/21/86	9/8/86	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		.25	.25	8/26/86	9/10/86	NEGATIVE
to Agar		.2	.2			NEGATIVE
Control Fluid - LOT # DENGUE 3 - QC 536						
PPLO Agar		.2	.2	8/11/86	8/26/86	NEGATIVE
PPLO Broth		.25	—			NEGATIVE
D 5 Subpass to Broth		.25	—	8/18/86	9/2/86	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		.25	.25	8/21/86	9/8/86	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		.25	.25	8/26/86	9/10/86	NEGATIVE
to Agar		.2	.2			NEGATIVE

Positive Control (+): M. orginini Negative Control (-): FB 29101099

Date: 9/10/86 Signed: Vera Guarley

Ben Venue
Laboratories, Inc.

270 NORTHFIELD ROAD • P.O. BOX 46568 • BEDFORD, OHIO 44146-0568 • 216/232-3320
TELEX 810-427-2275 • BEN VENUE BDFD • PANAFAX 216/232-2772

October 30, 1986

Dr. Louis Potash
Flow Labs., Inc.
7655 Old Springhouse Rd.
McLean, Virginia 22101

Dear Dr. Potash:

This is to certify that the sterility tests have been completed on Dengue Virus Type I (non-attenuated), Western Pacific, 1984 Lot #1 and Dengue Virus Type III (non-attenuated) CH53489 Lot #1. Both lots were found to be sterile as of October 22, 1986.

Copies of sterility tests #S6240 PF and #S6241 PF have been enclosed for your records.

P.O. # 89808.

With Best Regards,

BEN VENUE LABORATORIES

Dorothy Dougherty
Manager, Microbiology

STERILITY TEST OF POWDERS
USP Membrane Filter Method

Date Sampled N/A
Date Received 10-7-86
No. of Samples Received/Tested 20

BVL Control No. S 6 241 PF
Product Dengue Virus Type 3 (Non-Attenuated)
Lot No. CH53489 Lot No. 1
Thioglycollate No. L6138
Soybean-Casein Digest No. L6139
Date of Test 10-8-86
Operators Vicki Gunter
N/A
Test Time 1315 to 1515

Sample Reconstituted with sterile H2O
Lot No. BCS6259
Reconstituted Volume 10 mL
Type of Membrane Filter Used 0.2um
Volume of Recon'd Sample Filtered 200 mL
Volume of Fluid Thioglycollate 100 mL
Volume of Soybean-Casein Digest 100 mL
Volume of 0.1% Peptone Wash (P6233) 600 mL

	Thioglycollate	SCD
No. of Tubes used for Sterility Sample	1	1
No. of Reconstitution Fluid Controls	1	1
No. of Filter Controls	1	1
No. of Blank Media Controls	1	1
No. of Air Sham Media Controls	1	1
No. of 0.1% Peptone Wash Controls	1	1
No. of Tubes used for Water Control	N/A	N/A
No. of Tubes used for 250ml filter funnel controls	1	1

RESULTS:	Samples	Controls	Checked by
Date Read	10-22-86	10-22-86	Kathy Wiles
Fluid Thioglycollate (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	
Date Read	10-22-86	10-22-86	Kathy Wiles
Soybean-Casein Digest (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	

On the basis of the above data Dengue Virus Type-3, BVL Lot No. CH53489
Customer Lot No. Lot #1 is Sterile and is Acceptable
as of 10-22-86.
Identification: N/A

Kathy Wiles
Bacteriologist/Senior Technician

D.F. Daugherty
Manager, Microbiology Department

COMMENTS: Steritest®

New
Revised X
Replaces 12/16/81
Date 4/24/85

Ben Venue Labs., Inc.
Bedford, Ohio 44146
BVL13

ST. JUDE CHILDREN'S RESEARCH HOSPITAL
332 North Lauderdale, P.O. Box 318
Memphis, Tennessee 38101
(901) 522-0300
Danny Thomas, Founder

October 13, 1986

Dr. Louis Potash
Flow Laboratories
7655 Old Springhouse Road
McLean, Virginia 22102

Dear Lou,

Enclosed are the results of the reverse transcriptase assays for the samples sent 10/6/86. I consider all samples to be negative. The slightly elevated levels of the samples and control tissue culture fluid over my growth media controls are probably due to contaminating nuclear DNA polymerases from cell debris and/or nonspecific binding of the ^{3}H dTTP with cellular protein.

Sincerely,

Allan

Allan Tereba, Ph.D.

AT:lmw

Enclosure

SAMPLE (50µl)	^{3}H dTTP Mg^{++}	Incorporated MN^{++}
1. Dengue-1, Strain Western Pacific 1974	1,581	2,395
2. Dengue-1, TCF (control fluid for above) clar. 10/6/86	1,493	1,754
→ 3. Dengue-3, CH 53489 Challenge Seed, Day 9 (25 Apr 1984)	1,693	2,394
→ 4. Dengue-3, TCF (control fluid for above) clar. 10/6/86	1,467	1,602
Controls		
50 µl growth medium	302	379
50 µl culture fluid from PR-A RSV infected cells (Mg^{++} reverse transcriptase)	245,443	224,054
10 µl culture fluid from Moloney MLV infected cells + 40 µl growth medium (Mn^{++} dependent reverse transcriptase)	520,009	2,434,753

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